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Transformation of spider neurotoxin gene with prospective insecticidal properties into hybrid poplar *Populus simonii* × *P*. *nigra*

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Abstract：In recent years，the pest insects on hybrid poplar *Populus simonii* × *P*. *nigra* broke out heavily，which caused great losses in forestry. In order to improve insect resistance of *P*. *simonii* × *P*. *nigra* and avoid pollution due to insecticides，the fused BGT gene consisting of the insecticidal toxin gene from the spider，*Atrax robustus*，and the C terminal of Cry Ⅰ A(b) gene from *Bacillus thuringiensis* was transferred into *P*. *simonii* × *P*. *nigra* by *Agrobacteriumm-mediated transformation system. The results of PCR and Southern blotting analyses showed that the insecticidal gene of BGT was integrated into the genome of* P. simonii × P. nigra. *The corrected mortality of the second instar of* Lymantria dispar *in 6 days and 9 days after they were fed with the transgenic poplars was* 37.0% *and* 92.6%，*respectively. Analysis of variance showed that there was significant difference in body weight between* L. dispar *larvae fed with the transformed poplars and those fed with untransformed poplars. The results indicated that the growth rate of* L. dispar *fed with the transgenic poplars was affected negatively.*

Key words：Hybrid poplar；gene transformation；insecticidal neurotoxin gene；insect control；*Lymantria dispar*；spider neurotoxin

1 INTRODUCTION

Hybrid poplar，*Populus simonii* × *P*. *nigra*，were planted during the 1970s in Heilongjiang Province in north-eastern China，and it became one of the most common and important tree species used in the forest shelter belt in North China (Yang *et al*.，2001). Several species of forest pest insects heavily attacked *P*. *simonii* × *P*. *nigra*. The severity of the attack threatens the survival of these shelter belts. Both chemical and biological methods were attempted to control these pest insects on *P*. *simonii* × *P*. *nigra*. However，these attempts showed only limited effect mainly because the concealed feeding habits of these insects and the narrow window of opportunity when the ovipositing adults can be reached by the control measures.

Improving resistance of trees to pest insects by traditional breeding and selection is difficult and takes a long time. Molecular genetic studies designed to understand the mechanisms of host resistance conferred by plant defense genes offer an alternative approach to initiate programs aimed at improving pest resistance in host trees (Klopfenstein *et al*.，1993). Genetic engineering can contribute to improve pest resistance in trees through direct transfer of efficient defense genes or by facilitating early selection through information gained on the roles of specific defense genes in host resistance to pests (Schumann and Klopfenstein，1993).

Three kinds of insecticidal genes have been transferred into poplar trees to control the insect pests in China and abroad to date. The most widely used gene is the δ-endotoxin gene from *Bacillus thuringiensis* (Bt). The second type of insecticidal gene used for gene transfer into poplar is proteinase inhibitor (PI). The third type of insecticidal gene is the insect-specific neurotoxin. Spiders use their venom to kill prey or defend themselves. The toxin from spiders changes the sodium or calcium ions passage in front of synapse (Eun *et al*.，2002). In this study，a fused gene with the insecticidal toxin gene from the spider，*Atrax robustus*，was transformed into *Populus simonii* × *P*. *nigra* by *Agrobacterium*-mediated transformation system. We

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hope that the fused gene can be successfully used in breeding programs aimed at the selection of new poplar genotypes resistant to major insect pests on poplar trees, especially *Populus simonii* × *P. nigra*.

2 MATERIALS AND METHODS

2.1 Gene, vector and Agrobacterium strain

The gene encoding the toxin from *A. robustus* was synthesized by the State Key Laboratory of Protein Engineering and Plant Genetic Engineering (SKLPE&PGE), Beijing, China in cooperation with Deakin Company according to the amino acid sequence of the toxin (Jiang *et al*. 1995, 1996a). The fused gene, BGT, comprised of the toxin gene from *A. robustus* and the C terminal of the Cry Ⅰ A (b) gene from Bt, and the binary expression vector of the BGT, pYHY, was kindly provided by SKLPE&PGE, Beijing. *Agrobarcterium tumefaciens* strain LBA4404 containing binary vector plasmid pYHY (Fig. 1) was used to transform the BGT gene.

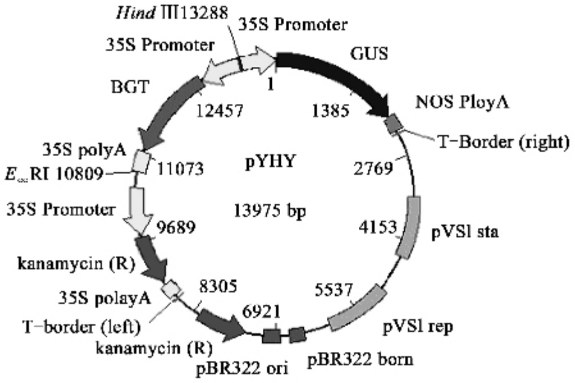

Fig. 1 The binary vector plasmid of pYHY

BGT of 1.3 kb was the fused gene of the toxin gene from *A. robustus* and the C terminal of the Cry Ⅰ A(b) gene from Bt. BGT gene was driven by cauliflower mosaic virus 35S promoter and 35S polyA was the terminator of BGT gene. Kanamycin (R) gene that was resistant to kanamycin was used for screening of transformed plants. GUS worked as a report gene.

2.2 Plants

P. simonii × *P. nigra* obtained from the campus of Northeast Forestry University (Harbin, China) was used as the source material for the plant transformation.

2.3 Transformation of *P. simonii* × *P. nigra*

The culture medium was prepared according to Murashige and Skoog (1962). The sterilized poplar leaves cut into segments of 0.5 cm × 0.5 cm were placed on the non-selective MS differentiation medium [MS + 0.5 mg·L^{-1} BA (N6-Benzyladenine) + 0.1 mg·L^{-1} NAA (a-Naphthalene acetic acid)] for 3 days. Then the pre-cultured leaf segments were taken from the MS differentiation medium and infected with *A. tumefaciens* LBA4404 containing pYHY for 2 min. The explants were placed onto sterile filter paper to remove excess bacteria, co-cultivated at 28℃ in darkness for 3 days on the MS differentiation medium, and then placed on the MS selective medium (MS differentiation medium + 50 mg·L^{-1} kanamycin) in 12L : 12D for regeneration of shoots. To limit the growth of *A. tumefaciens*, 500 mg·L^{-1} carbenicillin was added to the culture media. Antibiotics were filter-sterilized and incorporated into media after autoclaving. The medium was changed every 15 days. After shoots reached a height of 2 – 3 cm, they were separated and cultured on rootage medium consisting of half-strength MS macronutrients and 0.1 mg·L^{-1} NAA for root induction.

2.4 Preliminary screening of putative transformants

The leaves of the putative transformants and untransformed plants that worked as negative control were dipped in GUS detection liquid (Jefferson, 1987) at 37° C overnight to observe the expression of GUS gene.

2.5 Polymerase chain reaction (PCR)

Total poplar DNA was isolated from fresh leaf tissue in which GUS gene expression was positive using CTAB method of Rogers and Bendich (1988). Based on the sequence of BGT gene, a forward primer, 5′-cccatatgtctccaacttgcattc-3′, and a reverse primer, 5′-ggtctagattaatcaatatgataatccgtc-3′, were synthesized. The primers were synthesized at Sangon Company, Shanghai, China.

Polymerase chain reaction (PCR) was conducted in 50 μL reaction mixtures that contained 1 μL of each primer (25 pmol·μL^{-1}), 1 μL Taq polymerase (1.5 U·μL^{-1}), 5 μL 10 × Buffer solution [20 mmol·L^{-1} Tris-HCl (pH 8.4), 50 mmol·L^{-1} KCl, 1.5 mmol·L^{-1} $MgCl_2$], 4 μL dNTP (10 mmol·L^{-1}), 36 μL ddH_2O and 200 ng of poplar template DNA or 2 ng pYHY plasmid template DNA. A negative control in which ddH_2O water was used as template was included in PCR. This negative control demonstrated that the PCR reagents were free of contaminating template DNA. After preheating at 94℃ for 5 min, 35 PCR cycles were performed denaturing at 94℃ for 1 min, annealing at 55℃ for 1 min, extending at 72℃ for 1 min and BGT sequences were finally extended at 72℃ for 10 min.

PCR products were eletrophoresed on a 1% agarose gel and visualized in UV light after ethidium bromide staining.

2.6 Southern blot analysis of transgenic poplar plants

The plasmid pYHY was digested with *Bam*H Ⅰ and *Bst*E Ⅱ restriction endonucleases (Promega). Digested DNA samples were separated by electrophoresis on a 1% agarose gel. The probe with the protein coding region of BGT (*Bam*H Ⅰ - *Bst*E Ⅱ fragment of pYHY) was labelled according to the manufacturer's instructions of DIG DNA Labelling and Detection Kit (Boehringer). PCR products were transferred onto nylon membrane and hybridized with the probe to exclude the possibility of false positive PCR products.

2.7 Insect bioassay

Eggs of *L. dispar* were sterilized with 2% formalin solution for 3 – 5 min, then rinsed with sterilized water and air dried and placed in petridishes. The newly hatched *L. dispar* larvae were reared on artificial diets. Twenty and thirty newly molted (> 24 h) second instar larvae were placed onto the untransformed (the control) and transformed poplars, respectively. The seedlings were covered with nylon nets to prevent *L. dispar* larvae from escaping. The daily mortality of the second instar larvae was recorded 6 days and 9 days after treatment, respectively. The larvae fed on the control and transformed seedlings were weighed individually till the 6th day from the start of the bioassay using an electronic balance (BBI). The insect bioassay was repeated three times. Analysis of variance (ANOVA) (Tang and Feng, 2002) was used to evaluate the difference in body weight between the larvae fed on the control and those fed on transformed poplar leaves.

3 RESULTS

3.1 Regeneration of kanamycin-resistant explants

Within 20 days, green callus began to form at the cut ends of the explants inoculated with *A. tumefaciens* and cultured on MS selective medium containing 50 mg·L^{-1} kanamycin. Shoots also regenerated from some of these calli within 2 weeks (Fig. 2).

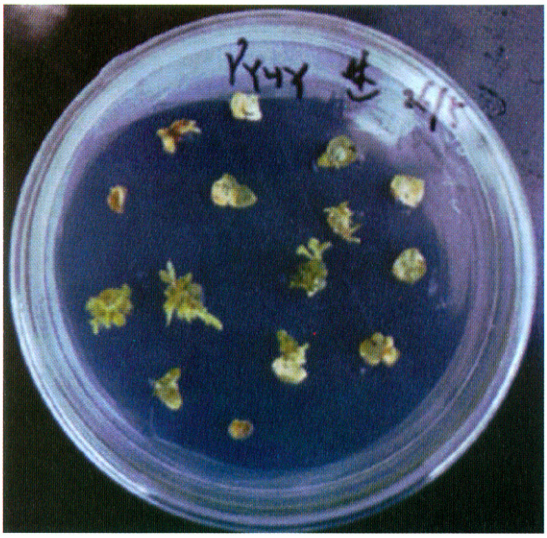

Fig. 2 The shoots regenerated from the callus of the transformed leaves cultured on the selective medium

Callus was not formed at the cut ends of the explants of untransformed poplars inoculated with *A. tumefaciens*. The explants of untransformed poplars turned yellow and died.

When the shoots grew up to 2 cm, the callus was removed from the base of the shoots and the shoots were transferred to fresh rooting medium. Roots were produced in 3 weeks (Fig. 3).

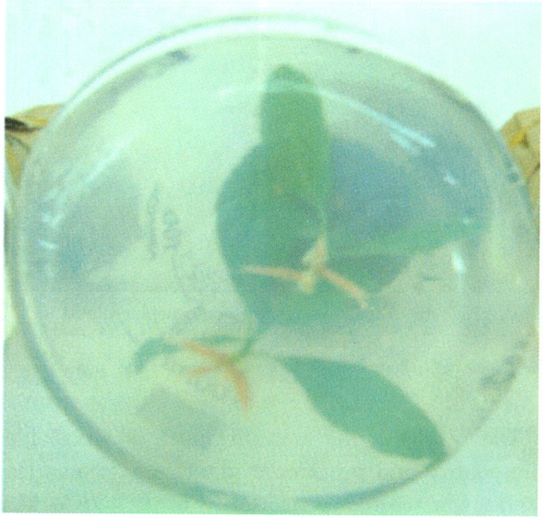

Fig. 3 The shoots cut from the transformed explants produced roots in root-induced culture medium (viewed from the bottom of a culture flask)

3.2 Expression of GUS gene

The leaves of the putative transformants dipped in GUS detection liquid became blue over night, indicating that the expression of GUS gene in the plants was positive (photo not shown here).

3.3 PCR analysis of the transformed poplars

The poplar plantlets with positive GUS expression were tested by PCR using primers specific for the BGT gene. Meanwhile, pYHY plasmid, water and an untransformed poplar plantlet were tested as controls. Six of the nine poplar plantlets showed the expected 1.3 kb band, whereas in the non-transgenic control this band could not be detected (Fig. 4).

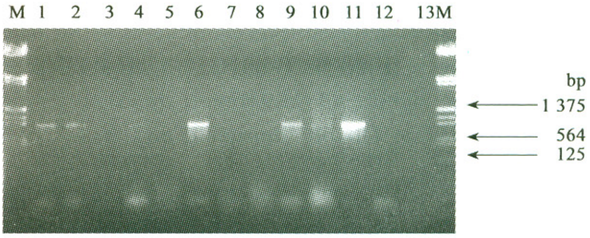

Fig. 4 PCR analysis of the transformed poplar plantlets

Lanes 1, 2, 3, 4, 5, 6, 7, 9, 10: GUS gene positive plantlets; Lane 8: PCR product of non-transformed plantlet; Lane 11, pYHY positive control (PCR product of pYHE); Lane 12: Negative control (water as PCR template); Lane 13: lambda DNA/*Hin*d Ⅲ + *Eco*R Ⅰ markers.

3.4 Southern analysis of the transformed poplars

PCR positive plantlets were selected for southern blot analysis. The hybridized bands were shown on the lanes of the transformed plantlets, whereas the non-transgenic control was negative, which confirmed the integration of the BGT gene into genomic DNA of transformed poplars (Fig. 5).

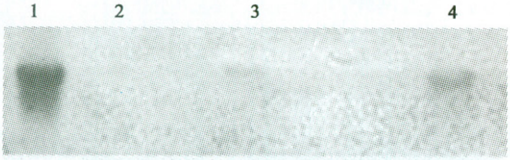

Fig. 5 Southern blot hybridization of transformed poplar plantlets

Lane 1: pYHE plasmid as a positive control; Lane 2: Untransformed plantlets as a negative control; Lanes 3 and 4: Transformed plantlets.

3.5 Bioassays on *L. dispar* larvae

The *L. dispar* larvae reared on transformed poplars consumed less leaves than those reared on untransformed plants (the control) on the 5th day from the start of the feeding bioassay (Fig. 6), which was visually estimated.

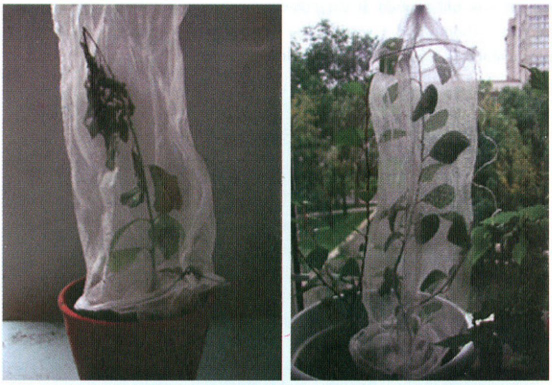

Fig. 6 Bioassays of transgenic poplar plants against *Lymantria dispar*

The photo was taken after 5 days of feeding. Left: non-transgenic plants; Right: transgenic plants

The results of the mortality rates of larvae feeding on the control and transgenic plants are summarized in Table 1. The corrected mortality of *L. dispar* larvae fed on the transgenic poplars was significantly higher than that of *L. dispar* larvae fed on non-transgenic poplars.

The result of ANOVA on body weights of the surviving larvae feeding on transgenic and control leaves on the 6th day is listed in Table 2, which showed that there was a significant difference in body weight between the larvae fed on transgenic and those on the control poplars.

Table 2 ANOVA on the body weight difference between *Lymantria dispar* larvae feeding on transgenic and control poplar leaves

Difference source	Sum of square	Degree of freedom	Mean square	KW	P
Model	23336.1111	1	2333.1111	20.7452	0.0001
Error	1538.3889	34	45.2467		
Total	38743.5000	35	110.7000		

In 9 days after treatment, *L. dispar* larvae were significantly smaller when feeding on transgenic leaves compared with control leaves. The larvae feeding on transgenic leaves were still in the second instar, while the larvae feeding on control leaves had already completed the third instar (Fig. 7).

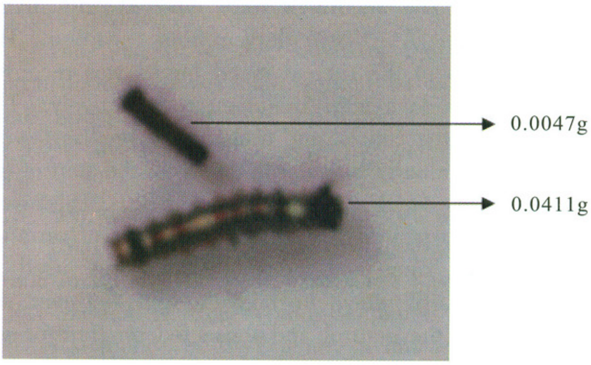

Fig. 7 The comparison of the larval size between the *Lymantria dispar* larvae fed on con trol and transformed plants.

The larvae in the figure hatched at the same time. They were as follows: the second instar larva (0.0047 g) fed on the transformed plants and the third instar larva (0.0411 g) fed on the control plants.

The above analysis clearly indicates that the development of *L. dispar* larvae feeding on transgenic poplars was retarded compared with that of larvae feeding on control poplar leaves. The average development time of the second instar larvae feeding on transgenic poplars was 15 days, which was 10.8 days longer than that of larvae feeding on non-transgenic (the control) poplar leaves.

4 DISCUSSION

The insecticidal toxin in this study is composed of 37 amino acids and was purified from the venom of *Atrax robustus* by Deakin Company in Australia. The toxin is capable of killing many pest insects, and it is not harmful to mammals (Jiang *et al.*, 1996a).

Table 1 The mortality of larvae feeding on the leaves of control and transgenic plants

	Days after treatment	Total larvae	Dead larvae	Mortality (% of initial)	Corrected mortality (%)
Control	6	20	2	10	
	9	20	2	10	
Transgenic plants	6	30	13	43.3	37.0
	9	30	28	93.3	92.6

Bt Cry I A (b) toxin gene is effective especially against lepidopteran insects. The signal peptide, the protein translated by the C terminal of Bt Cry I A (b) gene, can recognize the receptor cells in the midgut of lepidopteran insects, but has no toxic effect on the insects themselves (Aronson, 1993). The purpose of constructing BGT fused gene was to enhance the specificity of insecticidal proteins which could target midgut efficiently. It is expected that the BGT gene should have a special insecticidal effect on lepidopteran insects. Tobacco (*Nicotiana tabacum* L.) leaves was transformed with the insecticidal neurotoxin gene from *A. robustus* [not with C terminal of Bt Cry I A(b)]. The mortality of *Heliothis armigera* (Hübner) fed on transgenic plants was significantly higher (30% ~ 40%), and the growth of the survived insects was remarkably slower than that of larvae fed on the control plants (Jiang *et al*., 1996b). There have been no reports on bioassays using BGT gene or insecticidal gene cloned form spiders on the insects of other orders since then. The insecticidal neurotoxin gene was transformed into *P. simonii* × *P. nigra*. We assume that the insecticidal gene itself could be also resistant to coleopterous insect pests including longhorn beetles which can not be effectively controlled by traditional methods, and this will be investigated in our further investigations.

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向小黑杨转化蜘蛛杀虫毒素基因

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摘要：近年来，危害小黑杨 *Populus simonii* × *P. nigra* 的害虫发生严重，给林业生产造成很大损失。为了提高小黑杨的抗虫能力，避免使用杀虫剂带来的污染，用农杆菌介导法将澳大利亚漏斗蛛 *Atrax robustus* 的毒蛋白基因和苏云金芽孢杆菌 CryⅠA（b）基因 C 末端的融合基因 BGT 转化入小黑杨。PCR 和 Southern 印记分析转基因植株，结果表明，BGT 杀虫基因已经整合在小黑杨基因组上。活性实验表明，取食转基因杨树 6 天和 9 天后，舞毒蛾 *Lymantria dispar* 2 龄幼虫的校正死亡率分别是 37.0%和 92.6%。方差分析表明取食转基因和对照杨树的舞毒蛾幼虫体重差异显著。这些结果显示转基因杨树上的舞毒蛾的发育速率受到影响。

关键词：小黑杨；基因转化；杀虫毒蛋白基因；害虫防治；舞毒蛾；蜘蛛毒蛋白

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